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(54) Title: TRANSDUCTION VEHICLES FOR TRANSFERRING DNA TO A MAMMALIAN CELL

(57) Abstract

Transduction vehicles designed to transfer genetic material, especially to humans and human cells, processes of making the vehicles and processes of using them. The capsids of the vehicles are made from proteins that have the structures of naturally occurring viral capsid proteins. The vehicle's capsid proteins are synthesized in non-primate cells. The vehicle's capsid proteins are brought in contact with nucleoprotein complexes containing double-stranded DNA, thereby forming the vehicle.

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TRANSDUCTION VEHICLES FOR TRANSFERRING DNA TO A MAMMALIAN CELL

TECHNICAL FIELD

The inventions disclosed here are related to the construction of molecular vehicles, resembling viral particles, for accomplishing gene therapy.

BACKGROUND ART

Since the birth of gene manipulation technology, one of the major goals of biomedical research has been safe, specific, and beneficial gene therapy. Gene therapy can be used against infection, against cancer and to correct defective genes. It is currently under extensive study in many laboratories with favorable preliminary results. Indeed, the manipulation of genes, their promoters, enhancers and other regulatory elements is a relatively straightforward process in any competent molecular biology laboratory.

Transduction is a favored method for transferring nonviral genetic material to host cells. In transduction, the nonviral genetic material is packaged in viral capsids which are then allowed to contact the host cells in a manner that results in the introduction of the nonviral genetic material into the host cells.

Transduction vehicles have been constructed by others, using both RNA and DNA virus systems. In the present system, the capsids of mammalian DNA viruses are used to encapsidate the genetic material. Of particular interest, because their small size allows greater ease of construction, are the papovaviruses, a family composed of two genera, the polyomaviruses and the papilloma viruses.

Others have already contributed to the understanding of polyomavirus transduction vehicles. Oppenheim et al,(Proc. Natl. Acad. Sci., U.S.A., vol. 83, pp. 6925-6929 (1986)), working with SV40, a member of the polyomavirus genus, described a system composed of an SV40 ORI-containing vector, a helper SV40 virus whose purpose was to generate capsid proteins, and COS cells, which are an SV40-transformed monkey cell line, the COS cells providing SV40 T antigen needed for viral DNA replication. The system generated

pseudovirions in the COS cells; the pseudovirions could then be recovered and used to transduce or transfect human cells. (See also Oppenheim and Peleg, Gene, vol. 77, pp. 79-86 (1989), for systems for packaging SV40 pseudovirions and the risk of creating undesired infective recombinant virus.)

Another approach has been to start with empty polyoma capsids produced by infected mammalian cells, incubate the empty capsids with DNA, and then treat the capsid-DNA complexes with DNase so as to degrade DNA not actually within the capsid (S. N. Slilaty et al., J. Biol. Chem., 257:6571-6575 (1982)). When the capsids were initially incubated with polyoma DNA, however, not more than about half of any DNA molecule (i.e., about 1.8×10^6 daltons) was encapsidated - about half the size of the DNA found in naturally occurring polyoma.

A more successful approach, from the point of view of the size of the DNA molecule that was encapsidated, was taken by J. N. Brady et al (J. Virology, 32:640-647 (1979)) who started with polyoma produced by infected mammalian cells, and used a chelating agent and a reducing agent to disrupt the viral capsid into its capsomere components. Disruption also liberated the DNA-containing nucleoprotein that resided in the intact capsid. Brady et al disclosed that the capsomeres and nucleoprotein could apparently reassociate to form a viral particle. They speculated that their system could be used for gene therapy.

Of interest to the current invention was an observation made by D. M. Salunke et al (Cell, 46:895-904 (1986)) extending previous work of A. D. Leavitt et al (J. Biol. Chem., 260:12803-12809, (1980)). Salunke et al disclosed that if the major viral coat protein, VP1 was made in a nonmammalian cell, Escherichia coli, it could proceed to form capsomeres and capsid-like structures. The focus of their papers was not gene transfer, however, but rather the mechanism of capsid formation.

Despite the progress that has been made, there are still significant difficulties with current gene therapy technologies. Depending on the system, the problem can be that genetic information from pathogenic viruses is used, that the vehicle is made in mammalian cells that harbor pathogenic or

harmfully immunogenic agents not removed in the preparation of the vehicle, or that the large scale preparation of the vehicle is expensive because of the need for tissue culture systems for production.

The system of the current invention, when used in its most preferred mode, minimizes the above mentioned risks. In the current invention, viral capsid proteins can be made in a non-mammalian cell and mixed with a nucleoprotein particle containing the nonviral DNA sequences. The resulting vehicle can be used to transfer nonviral DNA to humans and the cells in their bodies. The amount of viral DNA used in the vehicle can be minimized so as to minimize the chances of recombination with any other viral genetic material unexpectedly present in the host cell. Mammalian cells are not required for the critical vehicle assembly step. Components of the vehicle, such as histones, that may originate from mammalian cells, can be highly purified before introduction into the vehicle. Furthermore, the fact that the capsid and DNA components can be produced in prokaryotic fermentation systems increases the cost efficiency of the process.

DETAILED DESCRIPTION

In one aspect, the invention is the process of constructing a transduction vehicle which process comprises the steps of

(1) synthesizing viral capsid proteins in cells that are not human cells or nonhuman primate cells, and (preferably while those capsid proteins are still disaggregated)

(2) allowing a nucleoprotein complex to come into contact with an amount of said viral capsid proteins sufficient to form a capsid enclosing said nucleoprotein complex,

said nucleoprotein complex containing a double stranded DNA molecule,

said viral capsid proteins corresponding in structure to proteins found in the capsid of a naturally occurring virus that has a double-stranded DNA nucleoprotein core. This process of constructing a transduction vehicle is

extended to another invention, a process of modifying a cell or organism, by having said process further comprise the steps of

(3) allowing the nucleoprotein complex to remain in contact with the viral capsid proteins for a time sufficient to result in the formation of a transduction vehicle comprising a capsid encapsidating the nucleoprotein complex, and

(4) contacting said transduction vehicle with a target, selected from a human cell, a non-human mammalian cell, a human or a non-human mammal. In a particular embodiment, the process comprises further a step (5) wherein the vehicle is permitted to remain in contact with its target for a time sufficient to allow the vehicle's DNA to enter said target.

If in step (4), the target is selected from a human cell or a non-human mammalian cell, a further aspect of the invention includes the further steps

5) permitting the vehicle to remain in contact with its target for a time sufficient to allow the vehicle's DNA to enter said target, and

6) if the target that has been contacted with the transduction vehicle is a human cell, administering said cell to a human, or, if the target that has been contacted with the transduction vehicle is a non-human mammalian cell, administering it to non-human mammal. Humans and human cells are considered the most common targets for the inventions.

In another aspect, the invention is a transduction vehicle comprising a capsid and a nucleoprotein complex, said nucleoprotein complex containing a double-stranded DNA molecule, the proteins of said capsid corresponding in structure to those in the capsid of a corresponding naturally occurring mammalian virus that has a double stranded DNA nucleoprotein core, provided that (1) any viral DNA in said DNA molecule does not contain viral ORI sequences, (2) said DNA molecule contains non-viral DNA, and (3) said double stranded DNA molecule of said vehicle is substantially enclosed within the capsid of said vehicle. The vehicle of the current invention could be called either a "transduction vehicle" or a "transfection vehicle", either term would be appropriat considering the vehicle is designed to be a viral-like particle useful for transmitting new genetic information to a cell.

A naturally occurring virus is one found in nature.

The "corresponding naturally occurring mammalian virus" refers to the naturally occurring mammalian virus that has capsid proteins with structures corresponding to those of the vehicle's capsid.

The term, "double-stranded DNA nucleoprotein core" refers to the core of a naturally occurring virus that is a nucleoprotein that contains a double stranded DNA molecule.

The capsid of a vehicle is the outer coat of the vehicle, just as the capsid of the virus is its outer coat.

"Said DNA substantially enclosed within said capsid" refers to the fact that the DNA will be enclosed by the capsid to an extent similar to the extent the viral DNA is enclosed by the capsid in the naturally occurring virus of similar capsid structure. This relationship between the capsid and the DNA offers maximum protection against enzymatic degradation of the DNA and also optimizes the probability that the DNA will, as in the naturally occurring virus, be delivered intact to the nucleus of a cell targeted for gene therapy.

An ORI sequence (ORI may alternatively be designated by others elsewhere as italicized ori, or simply as ori) is that base sequence at which a round of DNA replication is initiated on a double-stranded DNA molecule. In SV40, for example, the sequence is approximately 100 base pairs in length and its position on the SV40 DNA molecule has been mapped (K.N. Subramian and T. Shenk, Nucl. Acids Res. 5:3635 (1972); M.W. Gutai and D. Nathans, J. Mol. Biol., 126:259 (1978)). The actual initiation site of replication is at the G:C pair at position 0.67 within a 27 bp G:C rich region containing two 13 bp palindromes (In "Molecular Biology", D. Freifelder, Ed. p.884, Science Books International, Boston, 1983). The polyoma virus (Strain A2) ORI has been mapped close to the Bgl II site as described by B. E. Griffin and S. M. Dilworth (Adv. Cancer Res. 39:183 (1983)). Adenovirus has ORI regions at both ends of its linear DNA molecule ("Molecular Biology, D. Freifelder, Ed., Science Books International, Boston, 1983).

Another related invention is the process of carrying out gene therapy which process comprises the step of administering a transduction v hicle of this

invention to a human or other mammal. The vehicle may be first contacted with the cell of a human or other mammal and then the cell, now carrying the vehicle, may be administered or implanted in that human or nonhuman mammal.

In the inventions that are a transduction vehicle or are processes of either constructing a transduction vehicle, modifying a cell or organism, or carrying out gene therapy, it is preferred that the proteins of the vehicle's capsid correspond in structure to those of a corresponding naturally occurring mammalian virus, especially one that has a has a double-stranded DNA nucleoprotein core. The particularly preferred corresponding naturally occurring virus is either a papovavirus, an adenovirus or a herpes virus; even more preferred is that the correpsonding naturally occurring virus is a papovavirus or an adenovirus. In one embodiment of the process invention, the viral capsid proteins synthesized and contacted with the double stranded DNA nucleoprotein complex are all of the same protein species (e.g., all polyoma VP1 proteins).

In other embodiments of the vehicle and process inventions, the viral capsid proteins synthesized and contacted with the double stranded DNA nucleoprotein complex comprise the major viral capsid protein (e.g., the polyoma or SV40 VP1 protein); preferably they are a set of all the viral capsid proteins of the corresponding naturally occurring virus are (e.g., the polyoma VP1, VP2 and VP3 proteins).

It is preferred that the capsid proteins be synthesized in a non-mammalian cell, more preferably in prokaryotic cells or lower eukaryotic cells (e.g., a yeast cell).

Preferred conditions for the bringing the nucleoprotein in contact with the capsid proteins are presented in the Examples. More generally, preferred ranges are, 20°C to 37°C as to temperature, the equivalent of 100 to 250 mM NaCl as to ionic strength, 1×10^{-3} to 1×10^{-8} $CaCl_2$ as to divalent ions, and 7.0 to 8.0 as to pH, and 1:1 to 100:1 (w/w) as to the ratio of protein (capsid protein plus histone protein) to DNA. Less preferred, but possible for ionic strength are the equivalent of 50-100 mM or 250-500 mM NaCl.

The viral capsid proteins will correspond in structure to proteins which exist in the capsid of a naturally occurring mammalian virus. It is possible, however, that inconsequential [from the point of view that it does not prevent the construction of the transduction vehicle] amino acid substitutions and/or modifications in the length and/or additions via covalent linkage of chemical moieties to the amino acids of a capsid proteins will not prevent such a protein from carrying out its function for the present invention, its function being to form part of a capsid that protects the DNA of the transduction vehicle from nuclease action and/or allows the vehicle to bind to the membrane of a cell in a manner that ultimately results in the vehicle's DNA being present inside that cell. A protein that differs from a naturally occurring capsid protein only to the extent of such inconsequential substitution and/or modifications and/or additions is considered to be equivalent to such a capsid protein for purposes of the transduction vehicles of the present inventions.

Preferably, the capsid of the vehicle will be substantially the same, in terms of its three dimensional configuration, as that of the corresponding naturally occurring virus.

Elimination of the viral DNA ORI region further minimizes the chance of an undesired, unexpected recombinant viral DNA molecule forming during the process of gene therapy.

In one aspect of the invention, the DNA molecule of the transduction vehicle does not contain any mammalian viral ORI region. In another aspect, the DNA molecule contains a cellular DNA sequence that, when the vehicle's DNA is within a host cell's nucleus, can impart replication ability on the vehicle's DNA molecule so that it replicates in synchrony with the host cell DNA even when it is not integrated into the host cell's DNA.

It is preferred that the nonviral DNA of the vehicle contain the genetic information for at least one nonviral protein or nonviral regulatory nucleotide sequence; the purpose of the protein or nucleotide sequence being to affect the target cell in some desired fashion. Non-viral proteins include, but are not limited to, enzymes, hormones, neurotransmitters, antibodies, structural

proteins, repressor proteins, and operator proteins. Regulatory nucleotide sequences include, but are not limited to, enhancers, promoters and operators.

Pref rred transduction vehicles have double stranded DNA that corresponds to (or can be made to correspond to) a topological structure (e.g., a superhelical, closed circle in the case of SV40) and size to that in the capsid of the corresponding naturally occurring virus. However, DNA molecules smaller than the naturally occurring ones may be enclosed in the capsid (e.g., the SV40 capsid will enclose DNA that is 70 percent the size of normal SV40 DNA). The DNA molecule will preferably have sequences that allow it to replicate, for purposes of cloning and production, in cells relatively unrelated to mammalian cells (e.g., Escherichia or yeast).

Although it is preferred that the nucleprotein complex of the vehicle be substantially the same as that of the nucleoprotein core of the corresponding naturally occurring virus, regarding the nature of the proteins (e.g., the complex preferably has histones if the naturally occurring core has histones) and the number of the proteins per DNA molecule, as well as the size and topology of the DNA, there may be up to a 100 percent change in the actual nucleotide sequence of the DNA molecule.

It is preferred that the DNA of the vehicle be associated with nucleosomes or other protein structures that lead to a nucleoprotein structure that allows the packaging of DNA of a size substantially the same as that in the corresponding naturally occurring virus.

In further subgeneric aspects of the inventions, none of the transduction vehicle's DNA corresponds to a nucleotide sequence found in the naturally occurring virus with the same capsid proteins as the vehicle. When deciding whether a region of the vehicle's DNA molecule is viral DNA, regions less than about 10 base pairs in length should normally not be considered as the basis for the calculation. If the viral DNA is divided into segments that are too short (four base pairs, for example) even a random distribution of base pairs would make a nonviral DNA appear to have significant amounts of viral DNA.

The transduction vehicles of the current invention will be capable of interacting with a mammalian cell such that contact between the vehicle and

the cell will result in a sequence of events that lead to the DNA of the transducing vehicle appearing in the nucleus of said cell.

The transduction vehicles made by a process of the current invention will be capable of interacting with a mammalian cell such that contact between the vehicle and the cell will result in a sequence of events that lead to the DNA of the transducing vehicle appearing in the nucleus of said cell.

Choice of capsid structure

For a given vehicle, it is preferred that the capsid will correspond in structure, or be very similar in structure, to that of a naturally occurring virus. The choice of which type of capsid to construct will generally be dictated by the size of the DNA molecule to be transferred to the host organism. The smallest capsid that can be used to accommodate the vehicle's DNA molecule will usually be chosen as, the smaller the capsid, the easier it is to construct it. Accordingly, if the DNA molecule need not be more than about 3.5 million daltons, then usually an SV40-type or polyoma-type capsid structure will be selected. If the DNA molecule will be between about 3.5 and about 5 million daltons, then generally a papilloma virus-type capsid structure will be chosen. For DNA molecules between about 5 and about 22 million daltons, adenovirus-type capsids are preferred. For those between about 22 million daltons and about 100 million daltons, herpes virus-type capsids are preferred. Where the sum of the molecular weights of the required DNA segments of a molecule are less than the amount of DNA necessary to have capsid formation, the difference can be made up by including DNA sequences most likely harmless to the host organism. Candidates for such sequences are: those coding for a protein but either lacking translational and transcriptional start codons, or containing multiple stop codons in all reading frames; intervening sequence DNA lacking splice junctions; and, DNA sequences having no statistical correlation with any known genetic elements.

The papovavirus family (papovaviridae) is composed of two genera, the polyomaviruses and the papillomaviruses. This family is distinguished by a

naked (non-enveloped) icosahedral capsid and a circular double-stranded DNA genome of 3.5 to about 5 x 10^6 daltons. Simian virus 40 (SV40) normally packages a DNA molecule of about 5243 base pairs. The papillomaviruses normally package one of about five million daltons.

SV40 and polyoma have in common a number of properties that are relevant to the use of proteins with the structure of their capsid proteins in the transduction vehicles of the current invention: naked icosahedral capsids composed of 72 capsomeres; circular double-stranded DNA genomes of approximately 3.5 x 10^6 daltons; three viral capsid proteins encoded in their genomes; four cellular histones, H2A, H2B, H3 and H4 that are associated with the viral genome in the virus (this association yields nucleosomes and minichromosomes); early viral encoded proteins termed tumor antigens (SV40 has a large one (T antigen) and a small one (t antigen), polyoma has a large one, a middle t antigen, and a small one); and a dependence upon host cell proteins for viral transcription, translation and replication. The latest data suggests that the three SV40 encoded capsid proteins (VP1, VP2 and VP3) only associate as pentamers, but that some of these units function as though they were hexameric. Papillomaviruses also have a major capsid protein analogous to VP1, along with minor proteins that also appear to be involved in the capsid.

The papillomaviruses are reviewed in: The Papovaviridae, Vol. 2: The Papillomaviruses, N.P. Salzman and P. Howley, eds., Plenum Press, New York, 1987; A general review of the polyomaviruses is found in The Papovaviridae, Vol. 1: The Polyomaviruses, N.P. Salzman and P. Howley, eds., Plenum Press, New York, 1986. Included in Volume 1 is an article by J.N. Brady and N.P. Salzman that describes the general properties of the prototype polyomaviruses, polyoma and SV40 viruses. That article includes information on virion structure, viral DNA structure, viral proteins, viral infection, host range, chromatin structure, and virus assembly. It also identifies other known members of the polyomavirus genus: BK, JCV (human), K (mouse), RKV (rabbit), HaPV (hamster), STMV (stump-tailed macaque), LPV (Green

Monkey, and possibly also human) and SA12 (baboon). There may also be additional members of the genus.

Additional useful references in terms of polyomavirus properties are found in B.D. Davis et al, Microbiology, 2nd Edition, Ch. 63, Harper and Row, New York, 1973 and Virology, H. Fraenkel-Conrat and P.C. Kimbal, eds., pp. 186-193, Prentice-Hall, Inc., Englewood Cliffs, N.J., 1982. The usual size range of the genome suitable for packaging in SV40-type capsids is between 70 percent and just over 100 percent of the length of wild type SV40 DNA (See J.T. Elder et al, Ann. Rev. Genet., vol. 15, pp. 295-340 (1986); D. Ganem et al, J. Mol. Biol., vol. 101, pp. 57-83 (1976) and D. H. Hamer in Genetic Engineering, Vol. 2, J.K. Setlow and A. Hollaender, eds., pp. 83-101 (1980)).

African green monkey kidney cells (BSC-1, CV-1, etc.) are the most common permissive host for productive infection by SV40. Such cells are a preferred test system for certain properties of an SV40-type vehicle, especially those properties that are necessary for the DNA to reach the cell nucleus. There are also a number of non-permissive host cells in which the SV40 genome enters the cell, moves to the nucleus, usually integrates, is not replicated to yield virus particles, and frequently transforms the cell to the tumor state. These hosts include human cells and are discussed in Chapter 63 of Davis et al (reference cited above) and in Brady and Salzman (reference cited above).

Adenovirus capsids are composed of 240 hexon subunits and 12 penton subunits. There appears to be one type of viral encoded protein in the hexons, and two viral proteins in the pentons, comprising the penton base and fiber. The capsid is icosahedral and 600 to 900 angstroms in diameter. They normally package an adenovirus genome of 20 to 23 x 10^6 daltons, the molecular weight depending on the strain. Members of the adenovirus family include Group A (Types 12, 18 and 31), Group B (types 3, 7, 11, 14, 16 and 21) and Group C (Types 1, 2, 4, 5 and 6).

The herpesvirus family consists of viruses having capsids that range from 1800 angstroms to 2000 angstroms in diameter, have icosahedral symmetry and consist of 162 capsomeres. The capsomers are elongated hexagonal prisms

with a central hole. The herpesvirus genome is approximately 100 x 10^6 daltons. Members of the herpesvirus family include Herpes Simplex, Varicella - Herpes Zoster, Cytomegalovirus, Epstein - Barr Virus, monkey B virus, pig pseudorabies virus and others. Herpesviruses are enveloped.

Vectors or plasmids containing genes for various viral capsid proteins are available: For example, pBR SV, which has the entire SV40 genome molecularly cloned into pBR322, is ATCC number 45019. Additional molecularly cloned papovaviruses available through the ATCC are: BK virus, Bovine papilloma virus, Human papilloma virus, JC virus, K-virus, and polyoma strain A2 and A3 virus. Leavitt et al. (J. Biol. Chem. 260 (23):12803-12809 (1985) is a reference for cloned polyoma VP1. They can be introduced into any one of a number of prokaryotic and lower eukaryotic organisms. In actuality, the cloned proteins could be made in any cell, although from a practical standpoint it is preferable to use high efficiency fermentation systems. The capsid protein coding sequence must be preceded by a promoter sequence compatible with the cell in which it will be synthesized. Capsid proteins can be made, for example, in E. coli, B. subtilis, S. cerevisiae and other yeast, and lactobacillus. Cloning procedures for such systems are described in Sambrook et al. "Molecular Cloning: A Laboratory Manual", 2nd edition. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, NY.

DNA isolated from virus or infected cells can also be used as a source of DNA fragments from which to construct plasmids that will code for desired viral coat proteins.

Preparation of histone proteins

Histone chromosomal proteins ("histones") can be purified as monomers, dimers, octomers, and other supramolecular combinations from a variety of sources by a number of different methods. A preferred source is calf thymus because it is readily available and is of mammalian origin.

Calf thymus histones can be purchased commercially. (e.g., Boehringer Mannheim Corporation, Biochemical Products, 9115 Hague Road, P.O. Box 50414, Indianapolis, IN 46250-0414).

Procedures for the isolation and purification of histones from native sources for the assembly of nucleosomes in vitro are cited in A. Ruiz-Carrillo et al, Proc. Natl. Acad. Sci., U.S.A., vol. 76, pp. 3284-3288 (1979) for chicken histones and in J.E. Germond et al, Nucl. Acids Res., vol. 3, pp. 3173-3192 (1976) for calf thymus histones. Alternatively, by established techniques, the genes for these proteins can be introduced into E. coli or yeast, in a manner that will lead to the proteins being synthesized.

The level of histone acetylation can be adjusted for optimum assembly in vitro and/or expression in the target cells. This is indicated by the results of Salzman and Brady with SV40 (reference cited above) and also by the observations of M. Cotten and R. Chalkley (Nucl. Acids Res., vol. 13, pp 401-414 (1985)) in which histones that were hyperacetylated assembled into chromatin in vitro more efficiently than histones that were not. (See also, C.K.Shewmaker et al, Eur. J. Biochem., vol. 107, pp 505-510 (1980) regarding the possible beneficial effects of histone acetylation on the rapid onset of transcription.)

The level of histone acetylation may be altered in vivo through the use of sodium butyrate at millimolar concentrations (P.A. Egan and B. Levy-Wilson, Biochem., vol. 20, pp. 3685-3702 (1981)). It may be altered in vitro through the use of acetyladenylate (Shewmaker and Wagner, Eur. J. Biochem. 107:505-510 (1980) ; C. K. Shewmaker et al, Biochem. Biophys. Res. Comm., vol. 84, pp. 342-349 (1978)).

Although minichromosomes isolated from SV40 are formed from histones, H2A, H2B, H3 and H4, S.N. Sinha et al (Nucl. Acids Res., vol. 10, pp 5533-5552 (1982)) have found that nucleosome-like structures can be formed on SV40 DNA with non-histone chromosomal proteins. Therefore, it may be possible to assemble non-histone minichromosomes into the transduction vehicle capsids.

Assembly of DNA molecule

Cloning steps and manipulations may be performed according to standard protocols such as those described by Sambrook et al ("Molecular Cloning: A Laboratory Manual", 2nd edition. 1989. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

Preferably, the vehicle's double stranded DNA molecule has the size and topology (e.g., a supercoiled closed circle or a linear molecule) of the DNA that resides within the viral capsid in a naturally occurring virus.

For papovavirus-type capsids, it is preferred that the double stranded DNA molecule be a supercoiled, closed circle. The viral DNA isolated from intact papova viruses can be recovered initially in the form of a histone-containing nucleoprotein complex termed a minichromosome. When this minichromosome is stripped of protein, the viral DNA is recovered as a supercoiled double-stranded circular DNA.

Nucleosomes have nevertheless been formed in vitro on linear, relaxed DNA as shown by A. Ruiz-Carillo et al, Proc. Natl. Acad. Sci., U.S.A. vol. 76: pp. 3284-3288 (1979). A slightly different in vitro system uses relaxed circular DNA, containing a single stranded region as the substrate for nucleosome formation (S. Wittig and B. Wittig, Nucl. Acids Res., vol. 10, pp. 3647-3665 (1982)). Descriptions of the in vitro assembly of nucleosomes onto supercoiled SV40 DNA to form minichromosomes may also be found in J. E. Germond et al, Proc. Natl. Acad. Sci., U.S.A., vol. 72, pp. 1843-1847 (1976). Apparently, nucleosomes can be assembled not only onto linear DNA, nicked circular DNA and circular supercoiled DNA, but also covalently closed relaxed circular DNA - providing the histones are supplemented with a nicking closing activity such as a toposiomerase or a gyrase in combination with a ligase.

For Adenovirus-type and herpesvirus-type capsids it is preferred that the DNA be linear duplex DNA.

The nucleotide sequence of the vehicle's DNA will, of course, not be identical to that of DNA in the corresponding naturally occurring virus. At least some of its DNA will consist of genetic material which is of therapeutic

value to the host. Examples of such genes are described below under "Uses for the Vehicle". Frequently, the DNA will also carrier a reporter g ne, e.g. for chloramphenicol acetyl transferase (CAT) or for neomycin phosphotransferase (NPT).

Also present will be one or more nucleotide sequences required or advantageous for cloning and large scale production of the DNA in E. coli or other organisms. One such sequence is the E. coli ORI sequence. Another is one that codes for drug resistance. The use of the ORI and drug resistance genes have been reported on numerous occasions in the scientific literature. Procedures for constructing DNA molecules from different components and cloning them have been summarized elsewhere (Sambrook et al., ibid). Nucleic acids with a defined sequence can be purchased from commercial sources or made in the laboratory ("Oligonucleotide Synthesis: A Practical Approach", M. J. Gait, ed. IRL Press, Oxford., 1984).

Normally, the transduction vehicle's DNA does not possess the ability to replicate in a human host cell unless integrated into the DNA of said cell or unless said cell contains foreign DNA elements (elements not normally found in the cell) which provide helper functions that result in the replication of the transduction vehicle's DNA. To increase the probability of integration into the host cell's DNA, the DNA molecule of the vehicle can be modified to include sequences favoring such integration. It appears that the retroviral LTR sequences and at least one retroviral protein (IN) are involved in vivo and in vitro (F. D. Bushman et al., Science 249: 1555-1558, 1990; P. O. Brown et al.. Proc. Natl. Acad. Sci. USA 86:2525-2529, 1989; C. M. Farnet and W. A. Haseltine, Proc. Natl. Acad. Sci. USA 87:4164-4168, 1990; T. Fujiwara and K. Mizuuchi, Cell 54:497-504, 1988). The transduction vehicle would encode for the IN protein and any DNA sequences needed by the IN protein for integration. Efficient integration of the DNA of naturally occurring polyoma viruses appears to involve the DNA sequences containing the viral origin of replication and the presence of T antigen (Elder, J.T., Ann. Rev. Genet. 15: 195-340, 1981).

Normally, the transduction vehicle's DNA, unless it is integrated into the host cell's DNA (i.e., the target cell's DNA), will not have the ability to replicate in synchrony with that cell's DNA. Certain cellular DNA sequences might confer such a synchronous replication property on the vehicle's DNA even when the latter is not integrated into the host cell DNA.

Assembly of nucleoprotein molecule

This is illustrated by the specific examples given below. Generally, one strives to create conditions that physically and thermodynamically favor the correct association of protein with DNA. There are basically two ways this is done so as to form nucleprotein molecules such as nucleosomes. The first way is to start with purified components, such as histones and DNA, in high salt or other chaotropic conditions, and then slowly return the reaction mixture to more physiological conditions by dialysis and/or dilution with no salt or non-chaotropic buffers. The second way is to start with purified or partially purified components, such as histones, DNA, topoisomerase, and other factors possibly involved in an assembly process, and allow them to react in a physiological buffer (Wittig, S. and B. Wittig, Nucl. Acids Res.10 (12):3647-3665, 1982).

Encapsidation of nucleoprotein

Preferred conditions for encapsidation are illustrated in the Examples below. Generally, the process appears to be very similar to nucleoprotein assembly. In this case, however, the assembly process appears to be very dependent upon the pH and Ca^{++} composition of the buffer (Yuen, L. K. C. and R. A. Consigi, J. Virol. 43(1):337-341, 1982). In addition, the genome to be packaged should be somewhat similar to what is normally packaged in vivo, or should be convertible in vitro to that form.

Uses for the vehicle

The DNA of the vehicle may encode for proteins that will protect a person or animal from infective cells or viruses. Alternatively, it may encode for proteins that interfere with the replication or other functions of such infective entities; e.g. viral packaging or assembly. It also may perform an interference function by causing the synthesis of anti-sense nucleic acids that react with the messenger RNA or DNA of a virus. An anti-sense approach has been used to inhibit Rous Sarcoma virus (RSV) replication in cultured cells (Zamecnik, P. C. and M. L. Stephenson, Proc. Natl. Acad. Sci. USA 75:280-284, 1978)

The vehicles may be used against cancers mediated by retroviruses, other tumor viruses and oncogenes. Where the cancer is due to inappropriate expression of host genes, or is due to the "normal" expression of integrated viral genomes, gene therapy can be used to directly correct the gene defect or to repress the unwanted gene expression. In general, inappropriate gene expression is the result of either an operator/promoter sequence mutation that allows for derepression or activation of gene expression, or a mutation that alters the activity of a regulatory protein such that the gene is de-repressed or activated. The direct correction of this inappropriate expression could be by replacing the mutant control sequence with the correct control sequence; the corresponding indirect correction would entail a transduction vehicle encoded gene product that was modified to act in the required fashion upon the mutant control element. Alternatively, the direct correction could be by replacing the mutant gene encoding the altered transcription effector protein; the corresponding indirect correction would entail encoding the correct effector protein.

Anti-oncogene activity may consist of anti-sense RNA or DNA, or a repressor protein that acts on an oncogene promoter. A more extreme procedure would be to encode for a gene for a toxin that is only produced in transduced cells.

Possibilities for achieving permanent correction of defective host genes include gene replacement, correction, or complimentation. Defective host genes usually result in inappropriate host gene expression or a functionally defective protein. The gene therapy vectors may be engineered to replace the defective gene, to properly control gene expression, or to encode for a functional protein.

Additionally the vehicle can be used to deliver genes coding for polypeptide hormones.

In all of the above areas the vehicle presents a means not only for gene therapy but also for the testing and development of possible gene therapies.

A specific example of gene therapy would be to use the vehicle against herpes simplex virus (HSV) encephalitis. The gene responsible for this disease appears to be the HSV gamma$_1$ 34.5 gene (J. Chou et al, Science, vol. 250, pp. 1262-1266 (1990). The transduction vehicle could be used either prophylactically or after diagnosis to encode for a repressor of transcription of the gene, which contains a 500 bp region upstream of the gene that contains a late HSV promoter sequence. Alternatively, the transduction vehicle could be used to code for the synthesis of anti-sense nucleic acid. The use of anti-sense (complementary) RNA or DNA to alter gene expression is reviewed by A. R. van der Krol et al., Biotechniques 6(10):958-976, 1988. In general, the complementary nucleic acid is prepared such that a duplex will form with normally synthesized mRNA. This duplex is then either recognized as abnormal and degraded, prevented from being translocated across the nuclear membrane, or is incapable of serving as a template for translation. Hence that gene product is not produced. In the case of HSV gamma$_1$34.5, the transcript is approximately 800 bases long, so the transduction vector would contain this gene under the control of a target cell compatible promoter such that the complementary strand was produced by transcription. Shorter complementary mRNAs directed primarily towards the 5' end of the mRNA may also be suitable.

Another specific example of gene therapy is its use against Li-Fraumeni syndrome, a group of diverse tumors that has traceable familial ties and has

been correlated with gene defects in gene p53, a tumor suppressor (D. Malkin et al, Science, vol. 250, pp.1233-1238 (1990)). The syndrome has been correlated with gene defects both in somatic and germ cell lines. In this case, the vehicle would probably contain a nonmutant p53 gene, which codes for the p53 protein, which appears to contain a 393 amino acids (Fields, S. and S.K. Lang, Science 249:1046-1049, 1990) and therefore requires a coding sequence of approximately 1200 bp.

Other targets of gene therapy are cancers caused by defects in the retinoblastoma gene (Rb) (V. M. Ricciardi et al, Pediatrics vol. 61, p. 604 (1978), V.B. Franke et al, Cytogenetic. Cell Genet., vol. 24, p. 185 (1979)). The RB gene family encodes multiple polypeptides of 110 - 114 Kd which means a coding sequence of 3000 bp or less.

Another possibility of a vehicle encoding a suppressive gene is raised by the article of Weissman et al, Science, vol. 236, p. 175 (1987) in which introduction of a chromosome containing a non-mutant Wilms' tumor locus into a Wilms' tumor cell line corrected (suppressed) some of the malignant phenotype.

Still another target of gene therapy is cystic fibrosis. This is illustrated by the article, "Correction of the cystic fibrosis defects in vitro by retrovirus-mediated gene transfer" (M. L. Drumm et al, Cell, vol. 62, pp. 1227-1233 (1990)) along with two articles on cystic fibrosis gene therapy in cultured cells, R. J. Gregory et al, Nature, vol. 347, pp. 382-386 (1990) and D.P. Rich et al, Nature, vol. 347, pp. 358-363 (1990).

According to Riordan et al. (Science 245:1066-1073, 1989) the putative CFTR (Cystic Fibrosis Transmembrane Conductance Regulator) transcripts are approximately 6500 nucleotides in length. Given this size, the transduction vehicle with papilloma-type capsids, which has a genome of about 8000 bp, would be the preferred choice. The full length cDNA has been prepared in a plasmid, pTM-CFTR-3, as described by Gregory et al. (Nature 347:382-386, 1990) and has been shown to be very similar, if not identical to a protein found in airway epithelial cells but not fibroblasts.

Examples of situations where it is appropriate to first contact the transduction vehicle with a target cell and then administer the cell to a patient are the addition of genes for tumor necrosis factor to tumor-infiltrating lymphocytes and the subsequent administration of the cells to melanoma patients (currently being done by S. Rosenberg and coworkers at the National Cancer Institute(NCI)) and the addition of genes for adenosine deaminase (ADA) to T cells and the subsequent administration of these cells to patients with immune deficiency disease due to ADA deficiency (currently being done by W. F. Anderson and coworkers at the National Heart, Lung and Blood Insititute and by R. M. Blaese and coworkers at the NCI.)

Administration of the vehicle to patient

For the therapy to be totally effective, the therapeutic agent should be delivered in sufficient dose to each target cell. The current techniques for the transduction of target cells in vitro and in vivo include mechanical means such as microinjection, electroporation, and "projectile" devices.

Preferred means of administration are: via the blood system (e.g., by means of injection), and by local application, via a nasal route using aerosols or other sprays. Administration may, depending on the case, also be done by organ perfusion, catheterization through blood vessels to the target organ, orally, inhalation, direct injection into an organ or by absorption through the lower gastrointestinal tract.

EXAMPLES

The following examples are indeed intended to exemplify the inventions, not limit them.

Example 1

I. Histone Chromosomal Proteins.

Calf thymus histones H2a, H2b, H3 and H4 are purchased from Boehringer Mannheim Corp., Indianapolis, IN.

II. Viral Capsid Proteins

All cloning steps and manipulations are performed according to standard protocols such as those described by Sambrook et al ("Molecular Cloning: A Laboratory Manual", 2nd edition. 1989. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). The SV40 DNA used is the molecularly cloned "pBR SV" of G. Khoury and can be purchased under catalog number 45019 from the American Type Culture Collection. This plasmid contains the entire genome from SV40 strain 776 as a unique Bam HI fragment inserted into the unique Bam HI site of a pBR322 cloning vehicle.

The numbering system used to designate specific base pairs within the SV40 genome is that described by Buchman et al. ("DNA Tumor Viruses", 2nd edition revised, J. Tooze, ed. 1981. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. p. 799-841) in which the last "G" of the unique Bgl site in SV40 is designated as nucleotide number "one". A complete nucleotide sequence for SV40 strain 776 is available (Buckler, C.E. and N.P. Salzman, in "The Papovaviridae, Volume I: The Polyomaviruses", N.P. Salzman, ed. 1986. Plenum Press, NY, p. 379-394). The locations of the viral capsid proteins are given in this reference and in a review article by Brady and Salzman (Brady, J.N. and N.P Salzman, in "The Papovaviridae, Volume I: The Polyomaviruses", N.P. Salzman, ed. 1986. Plenum Press, NY. p. 1-26).

A. Cloning of VP1.

The nucleotide sequence encoding the SV40 capsid protein VP1 includes nucleotides numbered 1499-2590 and is contained within a 1199 bp

long Rsa I - Hpa I restriction fragment. Due to the nature of the pBR SV construct, the VP1 gene (protein coding sequence) is first isolated from pBR SV as two unique Bam HI-Rsa I restriction fragments of 539 bp and 1066 bp. These fragments are subcloned into the Bam HI and Hinc II restriction sites of a pT7/T3 alpha-18 cloning vehicle and transformed into E. coli NM522.

Transformations are plated in the presence of IPTG and X-gal in order to use blue/white screening to identify those clones with inserts. Mini-prep DNA from positive (white) colonies is prepared, digested with Bam HI and Sph I, and resolved by 1% agarose gel electrophoresis in 1X Tris-Borate-EDTA buffer to identify those clones having 551 and 1078 bp fragments, respectively. DNA from a clone identified as having the 1066 bp insert is digested with Sph I and then digested with nuclease Bal 31 for timed intervals to generate a set of progressive deletions moving in from the noncoding sequences 5' of the gene towards the AUG (start codon) of VP1. The deletion samples are further digested with Bam HI to liberate the fragment(s) containing the VP1 gene. A sample of this digest is subcloned into the prokaryotic inducible expression vector pKK223-3 (Brosius, J. and Holy, A., Proc. Natl. Acad. Sci. USA 81:6929, 1984) that has been digested with Eco RI, blunt-ended via a Klenow DNA polymerase I fill-in reaction, and subsequently digested with Bam HI. Transformations are into E. coli NM522. Miniprep DNA is prepared from the resultant colonies, analyzed for the correct insert, and DNA sequencing is performed to confirm insert identity and orientation. DNA from a pKK223-3 clone identified as having the correct VP1 insert is digested with Sal I, blunt-ended with Klenow DNA polymerase, and digested with Bam HI. A DNA fragment from a pT7/T3 alpha-18 clone identified as having the 539 bp VP1 insert is excised with Bam HI and Hpa I and subcloned into the blunt-ended VP1/pKK223-3 Sal I/Bam HI vector. Resultant clones are screened for the correctly sized insert and insert identity and orientation is confirmed by DNA sequencing.

B. Cloning of VP2.

The nucleotide sequence encoding the SV40 capsid protein VP2 includes nucleotides numbered 562 through 1617 and is contained within a 1068 bp long Nco I - Acc I restriction fragment. The VP2 gene is isolated from PBR SV by digestion with Nco I and Acc I, blunt-ended with Klenow DNA polymerase, and sub-cloned into Sma I digested pKK223-3. Resultant clones are screened for the correct orientation and insert identity is confirmed by DNA sequencing.

C. Cloning of VP3.

The nucleotide sequence encoding the SV40 capsid protein VP3 includes nucleotides numbered 916 through 1617 and is contained within a 754 bp long Mbo I - Acc I restriction fragment. The VP3 gene is isolated from PBR SV as a 1264 bp long Mbo I fragment. This fragment is purified from the gel and then digested with nuclease Bal 31 to generate a set of deletions of the non-coding sequences 5' upstream of the AUG (start) codon of VP3. The reaction products are digested with Acc I, and blunt-ended; the fragments are then subjected to by gel electrophoresis. Appropriately sized fragments are recovered from the gel and subcloned into Sma I digested pKK223-3. The resultant clones are screened for the correct orientation and insert identity is confirmed by DNA sequencing.

D. Growth of Bacteria and Preparation of Crude Extract.

Bacteria containing a construct coding for either VP1, VP2 or VP3 are grown and lysed essentially as described by Leavitt et al. (J. Biol. Chem. 260(23):12803-12809, 1985) and Salunke et al. (Cell 46:895-904, 1986). In brief, cells are grown to early log phase and induced with isopropylthiogalactoside (IPTG) to produce large quantities of the viral capsid

protein. Cells are harvested, lysed, and the lysates are partially purified by selective precipitation.

E. Purification of Cloned Viral Capsid Proteins.

Viral capsid proteins are purified individually from their respective cloned overproducing host strains essentially as described by Leavitt et al. (ibid). In brief, each lysate is prepared as described above, precipitated, and solubilized in 1 M NaCl, 50 mM Tris-HCl, pH 7.9, 5% glycerol, 2.0 mM EDTA, and 15 mM beta-mercaptoethanol. After dialysis into the buffer described above, but instead containing 100 mM NaCl and at pH 7.2, the sample is loaded onto a DE-52 (Whatman) diethylaminoethyl cellulose column equilibrated in the same 100 mM NaCl Buffer, pH 7.2. The viral capsid proteins elute in the 100 mM NaCl Buffer, pH 7.2 wash and are precipitated with ammonium sulfate. Each pellet is dissolved in 1 M NaCl Buffer, pH 7.2, dialyzed into 100 mM NaCl Buffer, pH 7.4 and loaded onto a P-11 (Whatman) phosphocellulose column equilibrated in the same 100 mM NaCl Buffer. The column is washed with 100 mM NaCl Buffer, pH 7.4 and the viral capsid proteins are eluted with a 100 mM - 1 M NaCl Buffer, pH 7.2 gradient. In all cases, the eluted fractions are monitored by A_{280} absorbance and the presence of the viral capsid proteins is confirmed by SDS-polyacrylamide gel electrophoresis and Western blot analyses. Peak fractions are dialyzed against 50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 0.01% Triton X-100, 20% dimethylsulfoxide and 15 mM beta-mercaptoethanol, and stored at +4 °C as a source of disaggregated capsid proteins.

III. Transfection Demonstration Plasmids.

A. pSV2 CAT Plasmid.

pSV2 CAT is a commercially available (ATCC Number 37155) transient gene expression plasmid with a double-stranded circular DNA of approximately

5003 bp in length. It contains the SV40 early region promoter, splice junction, 72 bp repeat enhancer, origin of replication, and transcript polyadenylation site cloned into a fragment of PBR322 containing the bacterial origin of replication and ampicillin resistance gene. Also within pSV2 CAT is the gene for chloramphenicol acetyltransferase (CAT) which serves as a reporter enzyme under the control of the SV40 early region promoter (Gorman, C. in "DNA Cloning: A Practical Approach", Volume II. 1985. D. Rickwood and B.D. Hames, eds. IRL Press, Washington, DC. p. 143-190).

IV. Assembly of Minichromosomes.

The plasmid DNA to be assembled with histones into minichromosomes is prepared by a modification of the alkaline lysis procedure (Birnboim, H.C. and Doly, J.. Nucl. Acid Res. 7:1513-1522, 1979) and purified by ion exchange chromatography (Plasmid Select™ spin column chromatography, 5 Prime -> 3 Prime, Inc.® , West Chester, Pennsylvania). The calf thymus histones obtained as described above are dialyzed at a minimum of 1 mg/ml into 20 mM Tris-Cl, pH 8.0, 2.0 M NaCl, 0.1 mM PMSF, 0.5 mM EDTA, and 5.0 mM DTT Assembly Buffer as described by Germond et al. (Nucl. Acids Res. 3 (11):3173-3192, 1976). Assembly of the DNA and the histones is performed essentially as described by Germond et al. (ibid.) and Wilhelm et al. (Nucl. Acids Res. 5 (2):505-521, 1978). In brief, the plasmid DNA and the 4 histones are added to the same siliconized tube at 200 - 400 ug/ml (microgram/ml) plasmid DNA and 200 - 400 ug/ml of the histone mixture in the presence of Assembly Buffer. The ratio of histone protein to plasmid DNA is 1:1 on a weight basis. This reaction mixture is incubated at 37°C for 1 hour. The reaction mixture is then sequentially diluted with no NaCl Assembly Buffer to the final NaCl concentrations listed below and incubated at 37°C for the times indicated.

Final NaCl Concentration	37°C Incubation Time
1.6 M	25 minutes
1.4	25
1.2	25
1.0	45
0.85	45
0.75	45
0.65	25
0.5	25
0.25	25

The complexes are separated from free histones by column chromatography essentially as described by Sinha et al. (Nucl. Acids Res. 10 (18): 5533-5552, 1982). The reconstituted minichromosome mixture is loaded in 0.25 M NaCl Assembly Buffer onto a gel filtration column (Sepharose Cl-4B (Pharmacia, Piscataway, NJ) or equivalent packing) equilibrated in the same Assembly Buffer. The minichromosomes are eluted with 0.25 M NaCl Assembly buffer while monitoring the A_{280} of the column effluent. Purified minichromosomes are stored, as recovered from the chromatography, on ice at about 4°C and are stable for several days.

V. Assembly of Pseudovirions (Transduction Vehicles)

The three viral capsid proteins and the in vitro assembled minichromosomes are prepared as described above. Assembly of these components into pseudovirions is done essentially as described by Brady et al. (J. Virol. 32 (2): 640-647, 1979) for dis-assembled polyoma virions. The minichromosomes and viral proteins are combined in an approximate protein weight ratio of 3 ug minichromosomes: 25 ug VP1 : 1 ug VP2: 1 ug VP3 in a pseudovirion assembly reaction buffer consisting of 50 mM Tris-Cl, pH 7.4, 150

mM NaCl, 1 mM EGTA, 0.01% Triton X-100, 15 mM beta-mercaptoethanol, and 5X 10^{-7}M $CaCl_2$ adjusted so that there is 1 mg/ml of VP1. The reaction mixture is incubated for 15 minutes at room temperature (21-22 °C) and then dimethylsulfoxide is added slowly (dropwise) to a final concentration of 20% (v/v). The reaction is mixed and incubated for 30 minutes at room temperature. The pseudovirion assembly reaction is dialyzed for 16 hours at about 4°C against 1X PBS, pH 7.4 containing 0.01% Triton X-100, 10% dimethylsulfoxide, and 5 x 10^{-7}M $CaCl_2$.

Pseudovirions are further purified by chromatography on a gel filtration column (Sephacryl S-1000 (Pharmacia)) equilibrated in 1X PBS, pH 7.4 and 0.01% Triton X-100. Chromatography is performed using the same buffer. Column effluent is monitored as above. The purified pseudovirions are stored, as recovered from the column, at about 4°C and are stable for several days.

VI. Transduction with Pseudovirions (Transduction Vehicles)

Since the assembled pseudovirions (transduction vehicles) are essentially infective, but non-reproducing in vivo, transduction of cultured monkey CV-1 cells is done in plates using a procedure derived from that described by Das et al. (Biochem. Biophys. Res. Comm. 89 (1):17-25, 1979). In brief, cells are grown in 100 mm plates to approximately 80% confluency in complete growth medium (MEM supplemented with 10% calf serum) under 5% CO_2. The growth medium is removed, the cells are washed with sterile 1X PBS, and 0.5 ml of a pseudovirion sample containing about 1.5 x 10^{-11}g of pseudovirions as measured by DNA content is added to the plate. Incubation is carried out for 15 minutes at 37°C and 5 ml of complete growth medium is added to each plate. Incubation is carried out at 37°C with CO_2 as needed for about 48 hours before assaying for transduction.

VII. Assays for Transduction.

A. Reporter Enzyme Analysis.

The expression and production of CAT protein in the transductants is determined by the commercially available double-antibody enzyme-linked immunoabsorbent assay (ELISA), "CAT ELISA for the detection and quantitation of Chloramphenicol Acetyltransferase (CAT) in crude cell extracts" kit available from 5 Prime -> 3 Prime, Inc.[R] Protocols for performing the assay are provided with each kit.

B. Transduction Efficiency.

The efficiency of transduction is determined by antibody staining and the subsequent visualization of the primary anti-CAT antibody with FITC-conjugated secondary antibody and fluorescence microscopy. The antibodies and an "Indirect immunofluorescence kit for Chloramphenicol Acetyltransferase" are commercially available from 5 Prime-> 3 Prime, Inc.[R] An example protocol for performing such assays on cultured cells is provided with each kit.

Example 2

The procedures are the same as for Example 1 except for the following modifications:

III. A. pRSV CAT Plasmid.

pRSV CAT is a commercially available (ATCC Number 37152) transient gene expression plasmid with a double-stranded circular DNA of approximately 5026 bp in length. It contains the Rous Sarcoma Virus (RSV) long terminal repeat (LTR), and the SV40 splice junction and polyadenylation

signal cloned into a fragment of pBR322 containing the bacterial origin of replication and the ampicillin resistance gene. Also within pRSV CAT is the gene for CAT which again serves as a reporter enzyme but this time under the control of the RSV LTR promoter (Gorman, C. ibid.).

Example 3

The example is carried out as in Example 1, except for the following modifications each of which is equally or less preferred than the one it replaces in Example 1:

I. A. Preparation of Calf Thymus Histones H2a, H2b, H3 and H4.

Nuclei and chromatin are prepared from freshly collected and rapidly frozen calf thymus gland using the method of Panyim et al. (J. Biol. Chem. 246 (13):4206-4215, 1971) as modified by Vandegrift et al. (Biochem. 13(25):5087-5092, 1974) and Shewmaker et al. (Biochem. Biophys. Res. Comm. 84(2): 342-349, 1978). (Fresh calf thymus is available commercially from Pel-Freez Biologicals.) Frozen calf thymus is broken into smaller pieces and placed into ice-cold homogenization buffer consisting of 0.25 M sucrose, 0.01 M $MgCl_2$, 0.01 M Tris-Cl, pH 8.0, and 0.1 Phenylmethanesulfonylfluoride (PMSF) at 20 ml of homogenization buffer per gram of tissue. The slowly thawing tissue is then homogenized briefly in a Waring Blendor or similar device. After filtering the homogenate through cheesecloth and/or Miracloth pre-wetted with homogenization buffer, the nuclei are pelleted by low speed (480 xg) centrifugation. The resultant supernatant is discarded. The nuclear pellet is gently resuspended in washing buffer consisting of 0.25 M sucrose, 0.01 M $MgCl_2$, 0.01 M Tris-Cl, pH 8, 0.1 mM PMSF and 0.3% Triton X-100 at 15 ml per gram of starting tissue. The nuclei are mixed gently in a Waring Blendor and re-pelleted for 2-3 wash cycles. The final supernatant is discarded, the nuclear pellet volume estimated, and the nuclear pellet is resuspended in 10 volumes of 0.025 M EDTA, 0.1 mM PMSF, and 0.1 M Tris-Cl, pH 8.0. The

nuclei are mixed gently in the Waring Blendor and centrifuged, as a wash step, and then the wash step is repeated. Th pellet is resuspended in 50 volumes of sterile deionized water and transferred to a "dounce" type homogenizer. The nuclei are homogenized gently until they are lysed. Lysis appears as increased solution viscosity and is confirmed by phase contrast microscopy. The chromatin is sheared by sonication and centrifuged at 16,000 xg to pellet debris.

The sheared chromatin supernatant is recovered for fractionation on hydroxylapatite as described by Simon and Felsenfeld (Nucl. Acids Res. 6(2):689-696, 1979). The sheared chromatin is dialyzed overnight into column equilibration buffer consisting of 0.63 M NaCl, 0.1 mM PMSF and 0.1 M potassium phosphate, pH 6.7. After dialysis, any insoluble material is pelleted by centrifugation at 16,000 xg. This material is loaded at 1 mg/ml DNA on an hydroxylapatite column equilibrated in column equilibration buffer, and the column is washed with the same buffer. The histones H2a, H2b, H3, and H4 are eluted with elution buffer consisting of 2.0 M NaCl, 0.1 mM PMSF, and 0.1 M potassium phosphate, pH 6.7. The peak fractions are pooled and dialyzed against several successive changes of 0.1 M Tris-Cl, pH 7.5, 2.0 M NaCl, 0.1 mM PMSF, 0.5 mM EDTA, and 5.0 mM DTT. All of the above steps are performed at about 4°C. The purified histones are stored on ice at about 4°C.

II. E. Purification of Cloned Viral Capsid proteins.

Viral capsid proteins are purified by immunoaffinity chromatography using purified antisera directed against SV40 virions. The purified polyclonal antibodies are immobilized onto rigid supports such as cyanogen bromide - activated or aldehyde-derivatized agaroses according to the protocols supplied by the matrix manufacturer. In this case, each capsid protein-containing lysate is prepared as described above, dialyzed into 1X PBS, pH 7.4 (8 mM Na_2HPO_4, 2 mM KH_2HPO_4, 150 mM NaCl, 3 mM KCl, pH 7.4) and loaded onto an immobilized anti-SV40 column equilibrated in the same buffer. The column is washed with 1X PBS, pH 7.4 and the bound viral capsid protein is

eluted with 0.1 M sodium carbonate, pH 10.4. The fractions are neutralized with 1 M Tris-Cl, pH 6.0. Protein purification is monitored as above. Peak fractions are dialyzed against 50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 0.01% Triton X-100, and 20% dimethylsulfoxide and stored at +4 °C.

IV. Assembly of minichromosomes

For plasmid DNA preparation, instead of Plasmid Select™ spin column chromatography, Qiagen resin (Qiagen™ resin, Chatsworth, California and Diagen GmbH, Dusseldorf, Germany) is used.

Instead of using the procedure of Sinha et al., the minichromosomes are purified by gradient centrifugation as described by Wilhelm et al. (ibid.) and Germond et al. (ibid.)

V. Assembly of pseudovirions

Instead of using Sephacryl S-1000, pseudovirions are purified by gradient centrifugation as described by Brady et al. (ibid.) and Salunke et al. (Cell 46: 8985-904, 1986).

Example 4

This example is carried out as is Example 1, except for the following modifications:

Chicken erythrocyte histones are used instead of calf thymus histones. Nuclei and chromatin are prepared from chicken blood using the procedure of Hewish and Burgoyne (Biochem. Biophys. Res. Comm. 52(2):504-510, 1973) as modified by Wilhelm et al. (Nucl. Acids Res. 5(2):505-521, 1978). In brief, the erythrocytes are collected by low speed centrifugation and resuspended in 10 volumes of 0.024 M EDTA, 0.075 M NaCl. The suspension is frozen and thawed to lyse the cells. The nuclei are pelleted and processing is continued as described in Example 2 for calf thymus nuclei. The sheared erythrocyte

chromatin is dialyzed overnight into equilibration buffer consisting of 0.7 M NaCl, 0.1 mM PMSF, and 0.1 M potassium phosphate, pH 6.7. The chromatin is chromatographed as described above, but using the higher salt equilibration buffer. The fractions are pooled, dialyzed and stored as described for calf thymus histones.

Instead of Plasmid Select™ spin column chromatography, two successive ethidium bromide-cesium chloride density gradient ultracentrifugations and column chromatography are used (Sambrook et al., "Molecular Cloning: A Laboratory Manual", 2nd edition, 1989. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

Example 5

Here the procedure of Example 1 is followed except that a plasmid with no viral DNA sequences is used instead of the pSV2 CAT plasmid.

Example 6

Here the procedure of Example 1 is followed, except that transduction of cultured cells, instead of being done with CV-1 cells, is done with Chinese Hamster Ovary (CHO) cells and complete growth medium is Hams F12 supplemented with 10% calf serum under 5% CO_2.

Example 7

Here the procedure of Example 1 is followed, except that transduction of cultured cells, instead of being done with CV-1 cells, is done with NIH3T3 cells and complete growth medium is DMEM supplemented with 10% calf serum under 10% CO_2.

WHAT IS CLAIMED IS:

1. A process of constructing a transduction vehicle which process comprises the steps of

(1) synthesizing viral capsid proteins in cells that are not human cells or nonhuman primate cells, and

(2) allowing a nucleoprotein complex to come into contact with an amount of said viral capsid proteins sufficient to form a capsid enclosing said nucleoprotein complex,

said nucleoprotein complex containing a double stranded DNA molecule,

said viral capsid proteins corresponding in structure to proteins found in the capsid of a naturally occurring virus that has a double-stranded DNA nucleoprotein core.

2. The process of Claim 1 wherein the corresponding naturally occurring virus is a mammalian virus.

3. The process of Claim 2 wherein the corresponding naturally occurring virus is either a papovavirus, an adenovirus or a herpes virus.

4. The process of Claim 3 wherein the corresponding naturally occurring virus is either a papovavirus or an adenovirus.

5. The process of Claim 4 wherein the corresponding naturally occurring mammalian virus is a papovavirus.

6. The process of Claim 5 wherein the corresponding naturally occurring mammalian virus is of the polyomavirus genus.

7. The process of Claim 6 wherein the corresponding naturally occurring virus is of the papillomavirus genus.

8. The process of Claim 1 wherein step (1) is done in a prokaryotic cell.

9. A process of modifying a mammal or mammalian cell which process comprises the steps of

(1) synthesizing viral capsid proteins in cells that are not human cells or nonhuman primate cells, and

(2) allowing a nucleoprotein complex to come into contact with an amount of said viral capsid proteins sufficient to form a capsid enclosing said nucleoprotein complex,

(3) allowing the nucleoprotein complex to remain in contact with the viral capsid proteins for a time sufficient to result in the formation of a transduction vehicle comprising a capsid encapsidating the nucleoprotein complex, and

(4) contacting said transduction vehicle with a target, selected from a human cell, a non-human mammalian cell, a human or a non-human mammal,

said nucleoprotein complex containing a double stranded DNA molecule,

said viral capsid proteins corresponding in structure to proteins found in the capsid of a naturally occurring virus that has a double-stranded DNA nucleoprotein core.

10. The process of Claim 9 wherein the corresponding naturally occurring virus is a mammalian virus.

11. The process of Claim 10 wherein the corresponding naturally occurring mammalian virus is either a papovavirus, an adenovirus or a herpes virus.

12. The process of Claim 11 wherein the corresponding naturally occurring mammalian virus is a papovavirus.

13. The process of Claim 9 wherein in step (4) the target is selected from a human cell or a non-human mammalian cell and the process comprises the further steps

5) permitting the vehicle to remain in contact with its target for a time sufficient to allow the vehicle's DNA to enter said target, and

6) if the target that has been contacted with the transduction vehicle is a human cell, administering said cell to a human, or, if the target that has been contacted with the transduction vehicle is a non-human mammalian cell, administering it to a non-human mammal.

14. A transduction vehicle comprising a capsid and a nucleoprotein complex, said nucleoprotein complex containing a double-stranded DNA molecule, the proteins of said capsid corresponding in structure to those in the capsid of a corresponding naturally occurring mammalian virus that has a double stranded DNA nucleoprotein core, provided that

(1) any viral DNA in said DNA molecule does not contain viral ORI sequences,

(2) said DNA molecule contains non-viral DNA, and

(3) said double stranded DNA molecule of said vehicle is substantially enclosed within the capsid of said vehicle.

15. The vehicle of Claim 14 wherein the corresponding naturally occurring mammalian virus is either a papovavirus, an adenovirus or a herpes virus.

16. The vehicle of Claim 15 wherein the corresponding naturally occurring mammalian virus is a papovavirus.

17. A process of gene therapy which process comprises the step of administering a transduction vehicle of Claim 14 to a human or non-human mammal.

18. The process of gene therapy which process comprises the steps of

(1) contacting a transduction vehicle of Claim 14 with a human cell or non-human mammalian cell and

(2) administering said cell, if it is a human cell, to a human or administering said cell, if it is a non-human mammalian cell, to a non-human mammal.

INTERNATIONAL SEARCH REPORT

International Appli ation No. PCT/US92/02000

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): C12N 15/87, 7/01; A61K 48/00
US CL : 435/172.3, 235.1, 320.1; 514/44

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	435/172.3, 235.1, 320.1; 514/44; 935/57, 62

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched[5]

Please See Attached Sheet.

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
X	US, A, 4,403,035 (Anderson et al) 06 September 1983, see entire document.	1,8
Y	Journal of Biological Chemistry, vol. 257, no. 11, issued 10 June 1982, Slilaty et al, "Polyoma-like particle: characterization of the DNA encapsidated in vitro by polyoma empty capsids", pages 6571-6575, see entire document.	1-6,8-13, 17, 18
Y	Science, vol. 220, issued 13 May 1983, Slilaty et al, "Gene transfer by polyoma-like particles assembled in a cell-free system", pages 725-727, see entire document.	1-6, 9-12
Y	Cell, vol. 46, no. 12, issued 12 September 1986, Salunke et al, "Self-assembly of purified polyomavirus capsid protein VP_1", pages 895-904, see entire document.	8
Y	Science, vol. 236, issued 08 May 1987, Selden et al, "Implantation of genetically engineered fibroblasts into mice: implications for gene therapy", pages 714-718, see entire document.	13, 17, 18

* Special categories of cited documents:[15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
03 June 1992	15 JUN 1992
International Searching Authority[1]	**Signature of Authorized Officer[20]**
ISA/US	MARY E. MOSHER, PH.D.

Form PCT/ISA/210 (second sheet)(May 1986) *

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

A	Proceedings of the National Academy of Sciences USA, vol. 83, issued September 1986, Oppenheim t al, "Efficient introduction of plasmid DNA int human hematopoietic cells by encapsidation in simian virus 40 pseudovirions", pages 6925-6929.	1-18
A	Journal of Virology, vol. 62, no. 6, issued June 1988, McLaughlin et al, "Adeno-associated virus general transduction vectors: analysis of proviral structures", p. 1963-1973.	1-18

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE[1]

1. ☐ Claim numbers _, because they relate to subject matter (1) not required to be searched by this Authority, namely:

2. ☐ Claim numbers _, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:

3. ☐ Claim numbers _, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING[2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee.

Remark on protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PREVIOUS SHEETS

II. FIELDS SEARCHED
ther Documents Searched:

APS, Biosis
Search terms: virus, viral, encapsidat?, encapsulat?, capsid, capsids, SV40, assembl?, vitro, packaging extract, papilloma?, pseudovirion, pseudovirions, polyoma, au= aposhian h?